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13. ABSTRACT *(Maximum 200 words)*

On the basis of an extensive body of cytogenetic literature as well as molecular evidence from our laboratory, we hypothesize that a tumor suppressor gene is located on the long arm of Human Chromosome 7 at the q31.1 band. Furthermore, we propose that inactivation of this tumor suppressor gene plays a role in the development of breast cancer.

The long term objectives of the study are to identify and clone the tumor suppressor gene and to determine its function. The objectives for the requested funding period are to provide functional evidence for the existence of this tumor suppressor gene in breast cancer using microcell fusion and to clone the DNA fragment containing the putative tumor suppressor gene using a novel approach based on the introduction of Yeast Artificial Chromosomes (YACs) into breast cancer cells. This novel approach will facilitate the cloning of putative tumor suppressor genes closing the technical gaps that exist between the identification of deleted regions of genome containing putative tumor suppressor genes and the actual cloning of DNA fragments containing the candidate genes.

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INTRODUCTION

Several reports in the literature indicate that karyotypic alterations of chromosome 7 are common in many different types of neoplasia including breast cancer (reviewed in Atkin and Baker, 1991; Pandis, et al. 1993). These alterations include monosomies, trisomies and deletions of different segments of the chromosome. More recently, several laboratories including our own have shown a high frequency of loss of heterozygocity (LOH) in the long arm of chromosome 7 suggesting the existence of a tumor suppressor gene in this region (reviewed in Zenklusen and Conti, 1996). Furthermore, microcell fusion experiments have shown that chromosome 7 can inhibit tumorigenicity or induce senescence in several cell lines (Zenklusen, et al. 1994b; Ogata, et al. 1993). On the basis of this cytogenetic, molecular and functional evidence, we hypothesize that a tumor suppressor gene is located in the long arm of chromosome 7 at the q31.1 band. Furthermore, we propose that inactivation of this tumor suppressor gene plays a role in the development of breast cancer.

The overall objective of these studies is to identify and/or clone this putative tumor suppressor gene using innovative approaches. Thus, we propose to do a number of experiments to develop this approach and to apply them to the cloning of this gene.

The proposed strategy consists of transferring the whole chromosome 7 as well as relevant fragment of this chromosome (as shown by our LOH studies) into breast cancer cell lines. Whole chromosomes can be transferred to the breast cancer cells by microcell fusion as described in previous experiments from our laboratory (Zenklusen, et al. 1994a). To transfer relevant fragments of chromosome 7 we propose to use spheroblast fusion with selected yeast artificial chromosomes (YACs) which contain the markers with higher LOH in breast cancer (Huxley and Gnirke, 1991; Markie, et al. 1993).

We have previously performed microcell fusion with MCF-7 cells obtaining slow growth cultures and abortive colonies. These results are similar to the ones obtained in Dr. Carl Barrett's laboratory (Ogata, et al. 1993) but differ from our previous results in rodent epithelial cells in which introduction of a chromosome 7 suppressed tumorigenicity with minimal effect on the in vitro proliferative potential (Zenklusen, et al. 1994a). Based on these results as well as data from our laboratory indicating that chromosome 7 can inhibit tumorigenicity in prostate cancer cells without affecting growth in tissue culture (Zenklusen, et al. manuscript in preparation), we postulate that two potential tumor suppressor genes may be present in chromosome 7, one affecting proliferation and the other affecting the relationship between the tumor cells and the host. To further explore this possibility we have carried out LOH studies in a number of well characterized breast cell lines using highly polymorphic microsatellite markers to determine the pattern of chromosome alterations in these cell lines and to choose other lines for future studies. We identify two lines that present a series of homozygous markers in the critical region, making them appropriate for these studies.

As we started the microcell fusion experiments, experiments in parallel performed in collaboration with Dr. Jean Claude Zenklusen and Dr. Eric Green (National Human Genome Research Institute, not funded by this grant) identified a very strong candidate gene. This is a new gene, without homology with any other known gene, which is widely expressed in both fetal

and adult tissues. Mutations have been identified in a number of cancer cell lines. Based on these findings, we concentrate our efforts in transfecting a retrofitted bacterial artificial chromosome (BAC) containing this gene (this BAC contains only this gene) and using as a control a BAC from the same region. The BACs have been transfected into two cell lines showing deletion in this region of chromosome 7 and several neo-resistant clones have been obtained from each cell lines and from each BAC. We are in the process of characterizing the clones and expanding to be injected into new mice to check for tumorigenicity. If requested by the DOD, we can submit an additional report in six month. In this report we expect to be able to provide definitive proof that the gene that we have cloned is the tumor suppressor gene in chromosome 7q31.

BODY

A. Experimental Procedures

BAC selection: In order to construct a single fully contiguous series (contig) of BACs, we utilized the YAC based physical map of chromosome 7 previously reported by Bouffard, et al. (1997). The entire region of interest, with boundaries at D7S522 and D7S677, is contained in a single YAC contig. Using the sequence-tagged sites (STS) contained in the YAC contig, a series of BACs were isolated and a minimal tiling path was selected spanning the whole region. In all, nine BACs (RG030h15, RG253b13,RG054j07, RG300c03, RG114a06, RG099b05, RG343p13, GS234b20 and RG068p20) were selected spanning about 1.2 Mb.

BAC retrofitting: The selected BACs were retrofitted to express a mammalian selection marker (*neo*) using the Cre-Lox system according to the protocol by Kim, et al. (1998). Briefly, 500ng of freshly prepared BAC DNA were mixed with 50ng of the retrofitting vector pRETRObac and 1 unit of Cre recombinase (Novagen, Madison, WI) in a total volume of 30µl of 1x reaction buffer. After incubation for 60 min. at 37°C, 5 min. at 70°C, 10 min. at RT and 60 min. at 4°C, the total reaction was drop-dialyzed for 3hrs against water on top of Millipore V membranes (13mm, 0.025 µm pore, #VSWP 01300) floated in a 24 well plates. The whole reaction was then mixed with 20µl of GeneHogs electrocompetent cells (Research Genetics, Huntsville, AL) and transferred to a chilled 0.1cm electroporation cuvette. The cells were pulsed at 1.8 kV, 200 ohms, 25µF using BioRad Gene Pulser (Hercules, CA). One milliliter of SOC media was then added and the cells were incubated for 60 min. at 37°C, after which aliquots of 200 and 400µl were plated in LB plates containing 50µg/ml of chloramphenicol and kanamycin, prespread with 50µl of 2% X-gal.

The colonies obtained were analyzed for the integrity of the insert by pulse field gel electrophoresis, and correct integration of the retrofitting vector was assessed by PCR as described by Kim, et al. (1998).

Transfection of large DNA fragments (BAC's) Transfections of the BAC's into the breast carcinoma cell lines was performed using the Fugene™ 6 transfection reagent (Boehringer Mannheim). This reagent is a non-liposomal blend which appears to have maximal levels of transfection with reduced toxicity for mammalian cells. Cells were transfected at 70%

confluency following the specification of the manufacturer. The range of plasmid used in these experiments was 1-3 ug.

Tumorigenicity experiments: Tumorigenicity will be evaluated as described in our previous experiments (Zenklusen, et al. 1994b). Briefly, 10 6 cells will be injected SC in the flank of Balb/c nude mice. Animals are kept until they developed tumors and need to be terminated according with Institutional and Federal guidelines. Tumor mass is estimated with a caliber weekly and recorded for each mouse. Five mice will be injected with each clone.

B. Results

As discussed in the Introduction section, previous experiments had shown that microcell fusion of MCF-7 cells produce abortive colonies. We hypothesize the existence of two suppressor gene is chromosome 7, one homologous to the one postulated by Oshimura and Barret that induce senescence or cell arrest and the other postulated by our laboratory in rodents cells as well as prostate carcinoma and other human tumors, did not alter cell growth in vitro but suppress tumorigenicity in vivo. In the previous report we have also shown that HOMOD analysis (homozygosity mapping of deletions; J. W. Fountain, personal communication) in breast carcinoma cell lines showed two different pattern in chromosome 7 (using contiguous markers of high heterogeneity). Thus, for our experiments we choose two cell lines that have a well defined minimal chromosomal fragment of LOH in our region of interest (D7S496-D7S490 and we have concentrated our efforts into these two cell lines. One of the cell lines, MDA MB231 is an Adenocarcinoma-derived line, and the other is ZR-75-1, a Ductal Carcinoma In Situ-derived line that contain a large area of homozygocity in the critical markers. Both lines have a similar pattern of homozygocity with 8 consecutive markers presenting the same allelic constitution. The probability that the patients were homozygous in this region is 0.000059; therefore we considered that these cell lines present LOH in the critical region and can be utilized for future experiments.

Also, during the last period we have continued our efforts to clone the gene by other conventional methods. For this purpose, we continue our collaboration with Zenklusen and Green (National Human Genome Research Institute). As a result of this collaboration the whole 1.2 Mb contig of the critical region was sequenced and putative genes were identified by Grail, and GeneScan analysis. Matching those predictions with ESTs known to be in the region (by virtue of their location in the GeneMap database or by PowerBlast matching to the dbEST database of the genomic sequence of the BACs) resulted in a complete picture of the genes in the region. Complete cDNAs for the previously unidentified genes were isolated and PCR assays were generated to amplify each exon from a panel of eight prostate/breast carcinoma cell lines for full sequencing. One of these novel genes presented mutations in 4 of the 8 cell lines analyzed. The gene that has been identified has fifteen exons and spans a 130 Kb segment of genomic DNA. The gene is contained in BAC H_RG114a06 and according with our computer prediction and EST content analysis, is the only gene in that BAC. The complete cDNA of this gene, in both directions and each of the two splice variants identified during the cDNA cloning process are being cloned into a mammalian expression vector at the moment. Mutations have been identified in these genes in several cell lines (see Table 1). Also to rule out the possibility

of a tissue culture artifact, we have sequenced 10 samples of colon cancer and we have identified 4 mutations in this set of tumors.

Based on these parallel results we decided to refocus the project in the BAC H_RG114a06 and in the two cell lines that have a bonafide identified deletion in this region. We carry out an experiment in which we transfect BAC H_RG114a06 into the cell lines MDA MB231 and ZR-75-1. As a negative control we transfect BAC H_RG343p13 which is distal to H_RG114a06, of similar size and contains the full Wnt2 gene which is known to be upregulated in breast tumorigenesis, making it a very unlikely TSG candidate.

To perform these experiments we test the sensitivity to G418 (neo) on the cell lines MDA MB231and ZR-75-1, and determine that optimal concentration for selection are 400 μg/ml for the MDA MB231, and for the ZR-75-1. Under these doses there is no viable cell in the culture after one week of exposure to neo.

Once optimal concentration of neo was determined, we proceed to transfect both cell lines with the selected BAC's. We tested several concentration of DNA and we found that we could get efficient colony formation (approximately 2-4 per plaque), at a concentration of 1μg in a plaque. Colonies were isolated using cloning cylinders and trypsin and expanded sequentially to 24 wells/plate and a six well plate. None of the colonies has presented any apparent impairment in growth and a selected number of colonies have been shown to have the neo markers as detected by PCR. A minimum of 5 colonies has been expanded for each cell line and for each BAC and they will be further characterized using microsatellite markers in that region. To detect tumorigenicity individual colonies will be injected in nude mice. We expect to be able to start injecting clones by the end of the current year and expect to have definitive results of the first clones in March. We expect that the whole experiment will be completed on May or June of 2000.

KEY RESEARCH ACCOMPLISHMENTS:

- By conventional positional cloning we identify a strong candidate gene for the suppressor gene in chromosome 7p31. Mutations were found in breast cancer cell lines as well as in prostate cell lines and samples from colon carcinomas.
- We establish a protocol for efficient transfectin of large fragments of DNA into breast cancer cell lines.
- We identify two prototype breast cancer cell lines with deletions in chromosome 7 q31, which were are being used to test the capacity of the gene to suppress tumorigenicity.
- We identify a BAC containing the putative tumor suppressor gene and transfect the BAC into two breast carcinoma cell lines. The same cell lines were transfected with an irrelevant BAC to serve as a negative control. This work is in progress. We expect that these experiments will provide proof of concept that tumor suppressor genes can be identified using transfection of large fragments of DNA corresponding to a region of high frequency of LOH.

REPORTABLE OUTCOMES: (List outcomes)

-Manuscripts: A manuscript describing this work is in preparation.

-Patents and licenses applied for/or issued: We have not applied for patents.
-Degrees obtained that are supported by this award: N/A
-Development of cell lines, tissue or serum repositories: Cell lines containing transfected BACs will be made available to the scientific community after the work is published.
-Informatics such as databases and animal models, etc: N/A
-Funding applied for based on work supported by this award: Funding to continue these experiments will be requested through a RO1 mechanism in the next NIH deadline.
-Employment or research opportunities applied for and/or received on experiences/training supported by this award: Two post-doctoral fellows and several technician receive training within this contract.

CONCLUSIONS

This project had two major goals: to develop techniques for functional cloning of tumor suppressor genes and to use this approach to clone a tumor suppressor gene in chromosome 7q31. Both of these goals have been achieved, although in some cases a change of strategy was necessary to adjust the project to technological changes. The first important change was that the whole region of interest was sequenced as part of the Human Genome Project. We collaborate with the group doing the sequencing and computer techniques were used to detect candidate genes. Therefore, the work of cloning was facilitated by this information.

The second strategic change was due to the realization that it was more feasible technically to transfer BAC's rather than YAC's as originally proposed. We have been able perform transfers of BAC's into mammalian cells in a reproducible manner and we have obtained colonies from two breast cancer cell lines. Cell lines were transfected with a BAC containing the putative tumor suppressor gene and an irrelevant BAC. The tumorigenicity assay of these clones are in progress. We expect that these experiments will provide the proof of concept that this method can be used to clone other tumor suppressor genes or at least narrow down rapidly extensive regions of the genome. Thus, we think that we have made subttancial progress to this goal of the project.

As discussed above we have identified a putative tumor suppressor gene. This gene is expressed in most adult tissues and mutations have been found in cell lines and samples of colon adenocarcinomas. We have continued our experiments of BAC transfection using a BAC containing this putative gene. The rationale for continuing the experiments focussed on the BAC H_RG114a06 containing the putative tumor suppressor gene is several fold:

- This particular BAC apparently has the tumor suppressor gene as the only gene. Therefore, if this BAC can suppress tumorigenicity, it will be very solid evidence that this gene is a bona fide tumor suppressor gene in this system because it will be able to suppress tumorigenicity when driven by its own promoter and is embedded in its own genetic background.
- It will accelerate the identification of the tumor suppressor gene because it can be transfected while the complete cDNA and splicing variants of the gene are determined. It also has the advantage that the gene delivered as a BAC will splice in a physiological manner and will have the splicing forms that are normally present in that particular tissue.
- The most important is that as discussed above, it will provide a proof of concept that tumor suppressor genes can be cloned by this functional strategy.

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Publications and Meeting Abstracts

One manuscript in preparation.

Personnel Receiving Pay From The Research Effort

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